Supplemental Protocol 3

Optimized Automated Phosphopeptide Enrichment SOP using Agilent NTA-polymeric resin

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NOTE: For this procedure to work, Bravo automation protocols for steps "A", "C" and "D"will need to be created for your lab based on automation steps provided at the end of the document.

A.   IMAC Cartridge Preparation (TIP)

Purpose

Remove shipping solution, strip iron charged resin packed in AssayMap Bravo cartridges, and recharge them with iron chloride.

NB: This protocol should be run on the same day that the IMAC cartridges will be used. Ideally, the protocol should be executed immediately before the **IMAC** enrichment protocol.

Preparation

1. Prepare TIPMIX01 (100mM EDTA)
2. Prepare TIPMIX02 (100mM $FeCl_3$)
3. Prepare TIPMIX03 (0.1% TFA)

Materials

- HPLC-grade water, JT Baker, Cat. No. 4218-03 {TIP-M01}
- 500mM EDTA, Sigma-Aldrich, Cat. No. E7889-100ML {TIP-M02}
- $FeCl_3$, Sigma-Aldrich, Cat. No. 451649-1G {TIP-M03}
- Fe-NTA Agarose AssayMap Bravo Cartridges, Agilent Technologies {TIP-M04}
- Trifluoroacetic acid (TFA), Sigma-Aldrich, Cat. No. T6508-25ML {TIP-M05}
- 500uL V-bottom plate, VWR, Cat. No. 89005-016 {TIP-M06}

- 96-Well Half-Area Flat Bottom Microplate, Greiner Bio-One, Cat. No. 675101 {TIP-M07}
- 1-Well Low Profile Reagent Reservoir, Axygen, Cat. No. RES-SW1-LP {TIP-M08}

Assets

- Agilent AssayMap-BRAVO Automated Liquid Handling Platform with VWorks 4 {TIPA01}

Reagent Mixes

ID	Name	Step	Composition	Volume/Well	Use
TIPMIX01	100mM EDTA	**TIP**	1:5 dilution of 500mM EDTA {TIPM02} in HPLC-grade water {TIPM01}	100 µL	Strips nickel from Ni-NTA charged agarose.
TIPMIX02	100mM $FeCl_3$	**TIP**	16.2mg/mL $FeCl_3$ {TIPM03} in HPLC-grade water {TIPM01}	100 µL	Charges stripped NTA agarose cartridges with Fe^{3+}; CAUTION HIGHLY ACIDIC
TIPMIX03	0.1% TFA	**TIP**	0.1% TFA {TIPM05} in HPLC grade water {TIPM01}	N/A	To wash internal and external AssayMap parts during protocol; CAUTION ACID

Mix Preps and Mini-worksheets:

TIPMIX01 – 100mM EDTA (15mL)
1. Pipette 12mL of HPLC-grade water {TIP-M01} into a conical vial.   ❏
2. Pipette 3mL of 500mM EDTA {TIP-M02} into the vial.   ❏

TIPMIX02 – 100mM $FeCl_3$ (15mL)

1. Weigh out at least 243mg of iron chloride {TIP-M03} to make at least 15 mL
2. Calculate amount of water to add in mL by dividing amount weighed out by 16.2

   ❏ Amount weighed: _____ mg
   ❏ Divide by: 16.2
   ❏ Water to add   _____ mL

TIPMIX03 – 0.1% TFA
3. Pipette 999 mL of HPLC-grade water {TIP-M01} into a 1L bottle.   ❏
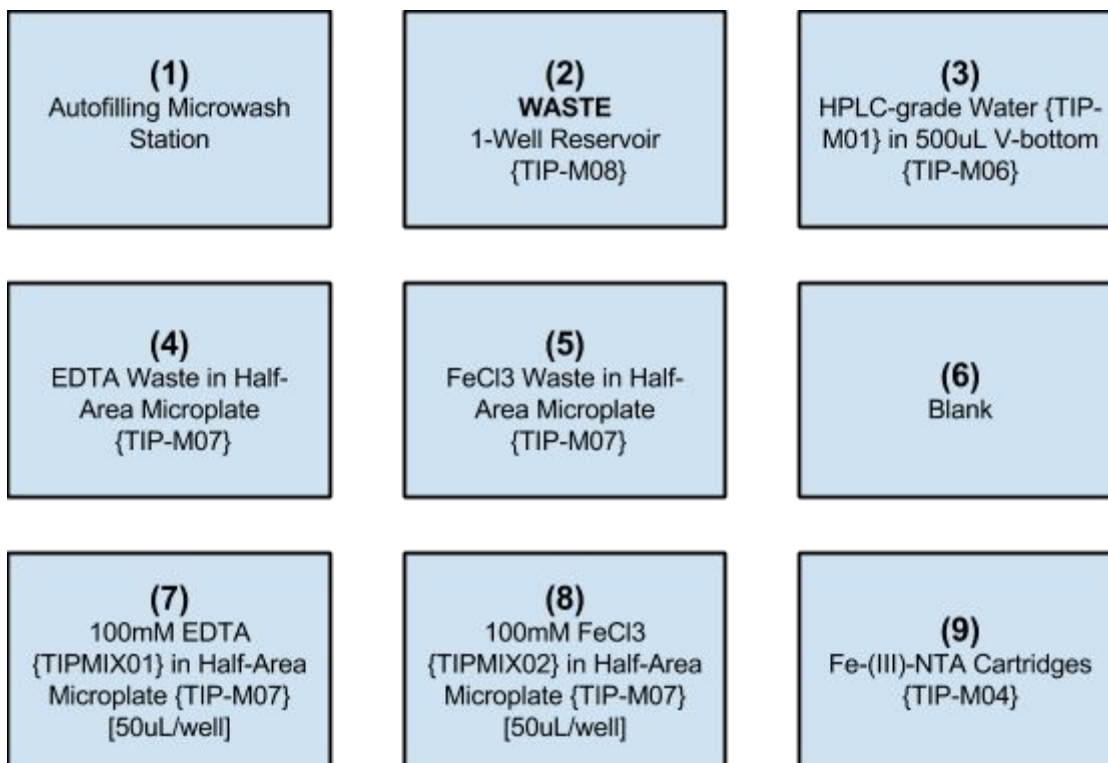4. Pipette 1mL of TFA {TIP-M05} into the bottle.   ❏

Procedure

1. Aliquot 100uL of 100mM EDTA {TIPMIX01} into each well of a 96-well half-area flat bottom microplate {TIP-M07} with a multichannel pipette.

2. Aliquot 100uL of 100mM FeCl$_3$ {TIPMIX02} into each well of a 96-well half-area flat bottom microplate {TIP-M07} with a multichannel pipette.

NB: 100mM FeCl$_3$ is very acidic and should be handled with care. Waste FeCl$_3$ needs to be recovered from the microplate at the end of the protocol and disposed of in the appropriate waste container found in the chemical hood.

3. Fill a 500uL V-bottom plate {TIP-M06} with 300uL [requires 225uL] of HPLC-grade water {TIP-M01}.

4. Prepare AM-BRAVO for operation:

    4.1. Fill the Wash Station reservoir with 0.1% TFA and ensure that the tubing is fully submerged. Check the waste container to see if the tubing leading into it is still above liquid level. If the container is full, dispose of the waste in the correct satellite waste container.

    4.2. On the AM-BRAVO Eva {TIP-A01} load the device file "AssayMap Bravo_CF.dev". This file is located at C:\\VWorks Workspace\Device Files\.

    4.3. In the "Devices" page, click on "Agilent Bravo" and then "AM-EVA". Select "Initialize all devices".

    4.4. Open the protocol file "1_pSTY_AgilentIMAC_Tip_Prep_CF_V2.pro". This file is located at C:\\VWorks Workspace\Protocol Files\AM-Bravo\IMAC\.

5. Assemble the deck of the AM-BRAVO according to the following layout:

(1) Autofilling Microwash Station	(2) WASTE 1-Well Reservoir {TIP-M08}	(3) HPLC-grade Water {TIP-M01} in 500uL V-bottom {TIP-M06}
(4) EDTA Waste in Half-Area Microplate {TIP-M07}	(5) FeCl3 Waste in Half-Area Microplate {TIP-M07}	(6) Blank
(7) 100mM EDTA {TIPMIX01} in Half-Area Microplate {TIP-M07} [50uL/well]	(8) 100mM FeCl3 {TIPMIX02} in Half-Area Microplate {TIP-M07} [50uL/well]	(9) Fe-(III)-NTA Cartridges {TIP-M04}

6. On the AM-BRAVO, toggle to "Simulation is on" at the top of the screen from "Simulation is off".

    6.1. Press Start and the Run Configuration Wizard will pop up. Press Finish.

    6.2. A pop up entitled "Set Initial Values for Variables" will appear. Set the number of "CartridgeColumns" to the appropriate amount of sample columns. (TIP Automation Protocol Step 1).

    6.3. Change the values for the other parameters listed if necessary and press ok.

6.4.   The simulation will run and provide feedback on any warnings or errors that the protocol may encounter. If there are any **unknown** errors that come up, notify the key AM-BRAVO user and obtain help. (There should be no errors when running this simulation).

7.   On the AM-BRAVO, toggle back to "Simulation is off". Follow steps 6.1 to 6.3 in order to run the protocol. (TIP Automation Protocol Steps 2-6).

8.   When the protocol is finished, do the following:

8.1.   Carefully check the IMAC cartridges for "iron charging"- they should have a faint yellow tint.

8.2.   Label IMAC tips located at the four corner positions with A1, A12, H1 and H12.

8.3.   Keep the IMAC cartridges on the deck or place at 4°C if not continuing immediately on to **IMAC Protocol**.

8.4.   Recover all $FeCl_3$ and dispose in the appropriate waste container in the chemical hood.

8.5.   Dispose of waste in the "Waste" reservoir at the appropriate satellite accumulation station.

8.6.   Retain any labware that can be reused. Empty appropriately, rinse with water, and leave to dry.

1.1.

B.   Sample Resuspension (RES)

Purpose

Resuspend dried peptides in 80% acetonitrile/0.1% Trifluoroacetic acid and spike in medium tagged phosphopeptide standards to track IMAC enrichment efficiency.

NB: Samples should be resuspended while the **TIP** protocol is running.

NB: Keep samples on ice until transferring to **IMAC** protocol.

Preparation

1.   Prepare RESMIX01 (50% ACN/0.1% TFA)
2.   Prepare RESMIX02 (100% ACN/0.1% TFA)
3.   Prepare RESMIX03 (80% ACN/0.1% TFA)
4.   Prepare RESMIX04 (Phosphopeptide Standards)

Materials

- HPLC-grade water, JT Baker, Cat. No. 4218-03 {RES-M01}
- Acetonitrile, EMD Millipore, Cat. No. AX0156-1 {RES-M02}
- Trifluoroacetic acid (TFA), Sigma-Aldrich, T6508-25ML {RES-M03}
- Concentrated Medium-tagged Phosphopeptide Standard Mix {RES-M04}
- 96-Well Round Bottom Microplate, Greiner Bio-One, Cat. No. 650101 {RES-M05} [650101]

Assets

- Sonicator {RES-A01}

Reagent Mixes

ID	Name	Step	Composition	Volume/Well	Use
RESMIX01	50% ACN/0.1% TFA	**RES**	50% acetonitrile {RES-M02}/0.1% TFA {RES-M03} in HPLC-grade water {RES-M01}	100uL	Resuspend dried sample peptides.
RESMIX02	100% ACN/0.1% TFA	**RES**	100% acetonitrile {RES-M02}/0.1% TFA {RES-M03} in HPLC-grade water {RES-M01}	140uL	Adjust acetonitrile concentration of resuspended peptide solutions to 80%.
RESMIX03	80% ACN/0.1% TFA	**RES**	80% acetonitrile {RES-M02}/0.1% TFA {RES-M03} in HPLC-grade water {RES-M01}	N/A	Create 1:50 dilution of medium phosphopeptides.
RESMIX04	1:50 Dilution of Concentrated Medium-tagged Phosphopeptide Standard Mix {RES-M05}	**RES**	1:50 dilution of concentrated medium phosphopeptides in 80% ACN/0.1% TFA {RESMIX03}	10uL	Track enrichment efficiency of IMAC cartridges.

Mix Preps and Mini-worksheets:

RESMIX01 – 50% ACN/0.1% TFA
1. Measure 500mL of HPLC-grade water {RES-M01} in a graduated cylinder and add to a 1L bottle. ❑
2. Measure 500mL of acetonitrile {RES-M02} in a graduated cylinder and add to the bottle. ❑
3. Pipette 1mL Trifluoroacetic acid {RES-M03} into the bottle. ❑

RESMIX02 – 100% ACN/0.1% TFA
1. Measure 999mL of acetonitrile {RES-M02} in a graduated cylinder and add to a 1L bottle. ❑
2. Pipette 1mL Trifluoroacetic acid {RES-M03} into the bottle. ❑

RESMIX03 – 80% ACN/0.1% TFA
1. Measure 200mL of HPLC-grade water {RES-M01} in a graduated cylinder and add to a 1L bottle. ❑

2. Measure 800mL of acetonitrile {RES-M02} in a graduated cylinder and
add to the bottle. ❑
3. Pipette 1mL Trifluoroacetic acid {RES-M03} into the bottle. ❑

RESMIX04 – 1:50 Dilution of Medium-tagged Phosphopeptide Standard Mix
1. Add 980uL of 80% ACN/0.1% TFA {RESMIX03} to 20uL of Concentrated
Medium-tagged Phosphopeptide Standard Mix {RES-M04}. ❑

Procedure

1. Add 100uL of 50% ACN/0.1% TFA to each dried peptide sample.
    1.1. Cover plate with foil, sonicate for 10 minutes, vortex thoroughly, and spin down.
2. Add 140uL of 100% ACN/0.1% TFA to each sample.
    2.1. Cover with foil, sonicate for 10 minutes, vortex thoroughly, and spin down.
    2.2. Samples will now be in 80% ACN/0.1% TFA.
3. Use a multichannel pipette to transfer each sample to a 96-well round bottom microplate {RES-M05}.
    3.1. The maximum volume this plate can hold is 250uL per well; samples should be 240uL each.
4. Add 10uL of diluted medium-tagged phosphopeptide standards in 80% ACN/0.1% TFA {RESMIX04} to each sample using a repeating pipette.

NB: Use caution while handling the plate. Do not seal the plate with foil.

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5. Place plate at 4°C until ready to continue to **IMAC** enrichment.

C. Automated IMAC Enrichment (IMAC)

Purpose

To enrich phosphopeptides via immobilized metal affinity chromatography (IMAC) so that P100 peptide probes can be easily quantified via mass spectrometric analysis. ***NOTE: This protocol uses commercially available Agilent AssayMAP NTA cartridges.***

Preparation

1. Prepare IMACMIX01 (0.1% TFA)
2. Prepare IMACMIX02 (80% ACN/0.1% TFA)
3. Prepare IMACMIX03 (1M Potassium Phosphate, Monobasic)
4. Prepare IMACMIX04 (1M Potassium Phosphate, Dibasic)
5. Prepare IMACMIX05 (500mM Potassium Phosphate Buffer, pH 7)
6. Prepare IMACMIX06 (1:1:1 Priming Buffer)

Materials

- HPLC-grade water, JT Baker, Cat. No. 4218-03 {IMAC-M01}
- Acetonitrile, EMD Millipore, Cat. No. AX0156-1 {IMAC-M02}

- Methanol, Fisher Scientific, Cat. No. A456-1 {IMAC-M03}
- 0.01% Acetic Acid, diluted from EMD Millipore, Cat. No. AX0074-6 {IMAC-M04}
- Potassium Phosphate Monobasic, Sigma-Aldrich, Cat. No. P0662-500G {IMAC-M05}
- Potassium Phosphate Dibasic, Sigma-Aldrich, Cat. No. P3786-500G {IMAC-M06}
- Trifluoroacetic Acid, Sigma-Aldrich, Cat. No. T6508-25ML {IMAC-M07}
- 500uL V-bottom plate, VWR, Cat. No. 89005-016 {IMAC-M08}
- 96-Well Half-Area Flat Bottom Microplate, Greiner Bio-One, Cat. No. 675101 {IMAC-M09}
- 96-Well skirted PCR plate, Bio-rad, Cat. No. MSP9601 {IMAC-M10}
- 96-Well Round Bottom Microplate, Greiner Bio-One, Cat. No. 650101 {IMAC-M11}
- Fe-NTA Polymeric AssayMAP Bravo Cartridges, Agilent Technologies G5496-60085 {IMAC-M12}
- Axygen -80°C Rated Foil Seal, Axygen, Cat. No. PCRAS200 {IMAC-M13}
- 1-Well Low Profile Reagent Reservoir, Axygen, Cat. No. RES-SW1-LP {IMAC-M14}

Assets

- Agilent AssayMAP-BRAVO Automated Liquid Handling Platform {IMAC-A01}

Reagent Mixes

ID	Name	Step	Composition	Vol/ Well	Use
IMACMIX01	0.1% TFA	**IMAC**	0.1% TFA {IMAC-M07} in HPLC grade water {IMAC-M01}	N/A	To wash internal and external AssayMap parts during protocol; CAUTION ACID
IMACMIX02	80% ACN/0.1% TFA (Binding Buffer)	**IMAC**	80% acetonitrile {IMAC-M02}/0.1% TFA {IMAC-M07} in HPLC-grade water	240uL	To equilibrate IMAC cartridges prior to sample loading. To wash IMAC cartridges prior to elution.
IMACMIX03	1M Potassium Phosphate, Monobasic	**IMAC**		N/A	Creation of IMACMIX05.
IMACMIX04	1M Potassium Phosphate, Dibasic	**IMAC**		N/A	Creation of IMACMIX05.
IMACMIX05	500mM Potassium Phosphate Buffer, pH 7 (Elution Buffer)	**IMAC**	500mM K_2HPO_4 in HPLC-grade water {IMAC-M01}	240uL	To elute phosphopeptides from IMAC cartridges.
IMACMIX06	1:1:1 Priming Buffer	**IMAC**	Acetonitrile {IMAC-M02}/Methanol	150uL	To activate IMAC cartridges prior to sample loading.

		{IMAC-M03}/0.01% Acetic Acid {IMAC-M04}		

Mix Preps and Mini-worksheets:

IMACMIX01 – 0.1% TFA
1. Measure 999 mL of HPLC-grade water {IMAC-M01} in a graduated cylinder and add to a 1L bottle. ❏
2. Pipette 1mL of TFA {IMAC-M07} into the bottle. ❏

IMACMIX02 – 80% ACN/0.1% TFA
1. Measure 20mL of HPLC-grade water {IMAC-M01} in a graduated cylinder and add to a 1L bottle. ❏
2. Measure 80mL of acetonitrile {IMAC-M02} in a graduated cylinder and add to the bottle. ❏
3. Pipette 100uL Trifluoroacetic acid {IMAC-M07} into the bottle. ❏

IMACMIX03 – 1M Potassium Phosphate, Monobasic
1. Weigh out at least 13.609g of potassium phosphate monobasic to make at least 100 mL
2. Calculate amount of water to add in mL by dividing amount weighed out by 0.13609
   ❏ Amount weighed: _____ g
   ❏ Divide by: 0.13609
   ❏ Water to add _____ mL
3. Test the pH of the solution, it should be around 4.

IMACMIX04 – 1M Potassium Phosphate, Dibasic
1. Weigh out at least 17.42g of potassium phosphate dibasic to make at least 100 mL
2. Calculate amount of water to add in mL by dividing amount weighed out by 0.1742
   ❏ Amount weighed: _____ g
   ❏ Divide by: 0.1742
   ❏ Water to add _____ mL
3. Test the pH of the solution, it should be around 10.

IMACMIX05 – 500mM Potassium Phosphate Buffer, pH 7
1. Pipette 9.625mL of 1M potassium phosphate monobasic {IMACMIX03} into a 100mL bottle. ❏
2. Pipette 15.375mL of 1M potassium phosphate dibasic {IMACMIX04} into the bottle. ❏
3. Pipette 25mL of HPLC-grade water {IMAC-M01} into the bottle, cap, and mix. ❏
4. Test the pH of the solution and ensure that it is at 7. ❏

IMACMIX06 – ACN/MeOH/0.01%AcOH
1. Pipette 6mL of 100% acetonitrile {IMAC-M02} into a conical vial. ❏
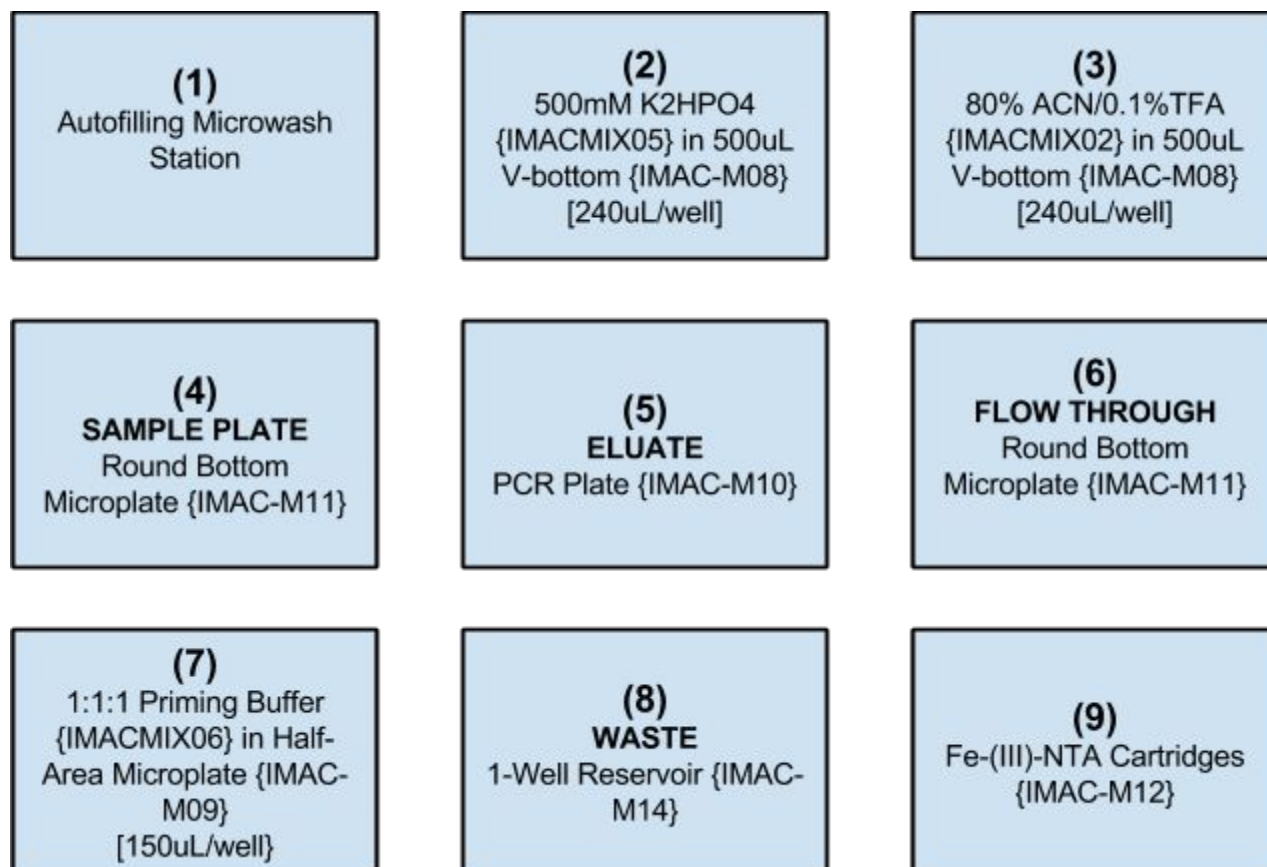2. Pipette 6mL of methanol {IMAC-M03} into the vial. ❏

3. Pipette 6mL of 0.01% acetic acid {IMAC-M04} into the vial. ❑

Procedure

1. Fill the Wash Station reservoir with 0.1% TFA {IMACMIX01} and ensure that the tubing is fully submerged. Check the waste container to see if the tubing leading into it is still above liquid level. If the container is full, dispose of the waste in the correct satellite waste container
2. Aliquot 150uL of 1:1:1 Priming Buffer {IMACMIX06} into each well of a 96-well half-area flat bottom microplate {TIP-M07} with a multichannel pipette.
3. Prepare AM-BRAVO for operation:
   - 3.1. On the AM-BRAVO Eva {IMAC-A01} load the device file "AssayMap Bravo_CF.dev". This file is located at C:\\VWorks Workspace\Device Files\.
   - 3.2. In the "Devices" page, click on "Agilent Bravo" and then "AM-EVA". Select "Initialize all devices".
   - 3.3. Open the protocol file "2_IMAC_Bind Wash Elute_CF_AgilentIMAC_V2.pro". This file is located at C:\\VWorks Workspace\Protocol Files\AM-Bravo\IMAC\.
4. Assemble the deck of the LT-BRAVO according to the following layout:

NB: Wait until the "Sample Loading" step has completed and the protocol has paused to fill and place the 80%ACN/0.1%TFA {IMACMIX02} and 500mM potassium phosphate buffer {IMACMIX05} on the deck.

(1) Autofilling Microwash Station	(2) 500mM K2HPO4 {IMACMIX05} in 500uL V-bottom {IMAC-M08} [240uL/well]	(3) 80% ACN/0.1%TFA {IMACMIX02} in 500uL V-bottom {IMAC-M08} [240uL/well]
(4) SAMPLE PLATE Round Bottom Microplate {IMAC-M11}	(5) ELUATE PCR Plate {IMAC-M10}	(6) FLOW THROUGH Round Bottom Microplate {IMAC-M11}
(7) 1:1:1 Priming Buffer {IMACMIX06} in Half-Area Microplate {IMAC-M09} [150uL/well}	(8) WASTE 1-Well Reservoir {IMAC-M14}	(9) Fe-(III)-NTA Cartridges {IMAC-M12}

5. On the AM-BRAVO, toggle to "Simulation is on" at the top of the screen from "Simulation is off".
    5.1. Press Start and the Run Configuration Wizard will pop up. Press Finish.
    5.2. A pop up entitled "Set Initial Values for Variables" will appear. Set the number of "CartridgeColumns" to the appropriate amount of sample columns. (IMAC Automation Protocol Step 1).
    5.3. Change the values for the other parameters listed if necessary and press ok.
    5.4. The simulation will run and provide feedback on any warnings or errors that the protocol may encounter. If there are any **unknown** errors that come up, notify the key AM-BRAVO user and obtain help.

6. On the AM-BRAVO, toggle back to "Simulation is off". Follow steps 7.1 to 7.3 in order to run the protocol. (IMAC Automation Protocol Steps 2-7).
7. **The protocol will pause after "Sample Loading" is complete.**
    7.1. Fill a 500uL V-bottom plate {IMAC-M08} with 240uL per well of 80%ACN/0.1%TFA {IMACMIX02} and place the plate in position 3.
    7.2. Fill a 500uL V-bottom plate {IMAC-M08} with 240uL per well of 500mM potassium phosphate buffer {IMACMIX05} and place the plate in position 2.
8. Upon completion of the protocol, note the condition of samples and seal plates to be saved.
    8.1. There will be residual volume in the **SAMPLE PLATE** in position 4.   Place foil seal {IMAC-M13} on the plate and transfer to -80C.
        ❏
    8.2. Place foil seal {IMAC-M13} on **FLOW THROUGH** plate in position 4 and transfer to -80C.
        ❏
    8.3. Carefully move the **ELUATE** plate from position 5 to position 4 and start with next protocol **Reversed Phase Peptide Desalt (RPS).**   ❏
9. Clear the deck.
    9.1. Dispose of waste in the "Waste" reservoir at the appropriate satellite accumulation station.
    9.2. Retain any labware that can be reused. Empty appropriately, rinse with water, and leave to dry.
    9.3. Label remaining IMAC Cartridges with date and project {IMAC-M12} and store at 4°C.

D. Reversed Phase Peptide Desalt (RPS)

Purpose

To remove salts from samples prior to mass spectrometric analysis.

Preparation

1. Prepare RPSMIX01 (0.1% TFA)
2. Prepare RPSMIX02 (50% ACN/0.1% TFA)

Materials

- HPLC-grade water, JT Baker, Cat. No. 4218-03 {RPS-M01}
- Acetonitrile, EMD Millipore, Cat. No. AX0156-1 {RPS-M02}
- Trifluoroacetic Acid, Sigma-Aldrich, Cat. No. T6508-25ML {RPS-M03}

- 500uL V-bottom plate, VWR, Cat. No. 89005-016 {RPS-M04}
- 96-Well Hard Shell, skirted PCR plate, Bio-rad, Cat. No. HSP9601 {RPS-M05}
- 96-Well Round Bottom Microplate, Greiner Bio-One, Cat. No. 650101 {RPS-M06}
- Agilent AssayMap Bravo RP-S Cartridges, Agilent Technologies, Cat. No. G5496-60033 {RPS-M07}
- Breathable Seal, Diversified Biotech, Cat. No. BERM-2000 {RPS-M08}
- Axygen -80°C Rated Foil Seal, Axygen, Cat. No. PCRAS200 {RPS-M09}
- 1-Well Low Profile Reagent Reservoir, Axygen, Cat. No. RES-SW1-LP {RPS-M10}
- 500uL Micronic Vials (Snap Tubes) in Loborack, Micronic, Cat. No. 1754-2072 {RPS-M11}

Assets

- Agilent AssayMap-BRAVO Automated Liquid Handling Platform with VWorks4 {RPS-A01}
- Thermo Scientific Savant SC210A Concentrator {RPS-A02}

Reagent Mixes

ID	Name	Step	Composition	Volume/Well	Use
RPSMIX01	0.1% TFA (Wash Buffer)	**RPS**	0.1% TFA {RPS-M03} in HPLC grade water {RPS-M01}	480uL [355]	Wash and equilibrate RP-S cartridges. CAUTION ACID
RPSMIX02	50% ACN/0.1% TFA (Elution Buffer)	**RPS**	50% acetonitrile {RPS-M02}/0.1% TFA { RPS-M03} in HPLC-grade water	480uL [350]	Elute peptides from RP-S cartridges.

Mix Preps and Mini-worksheets:

RPSMIX01 – 0.1% TFA
1. Measure 999 mL of HPLC-grade water {RPS-M01} in a graduated cylinder and add to a 1L bottle. ❑
2. Pipette 1mL of TFA {RPS-M03} into the bottle. ❑
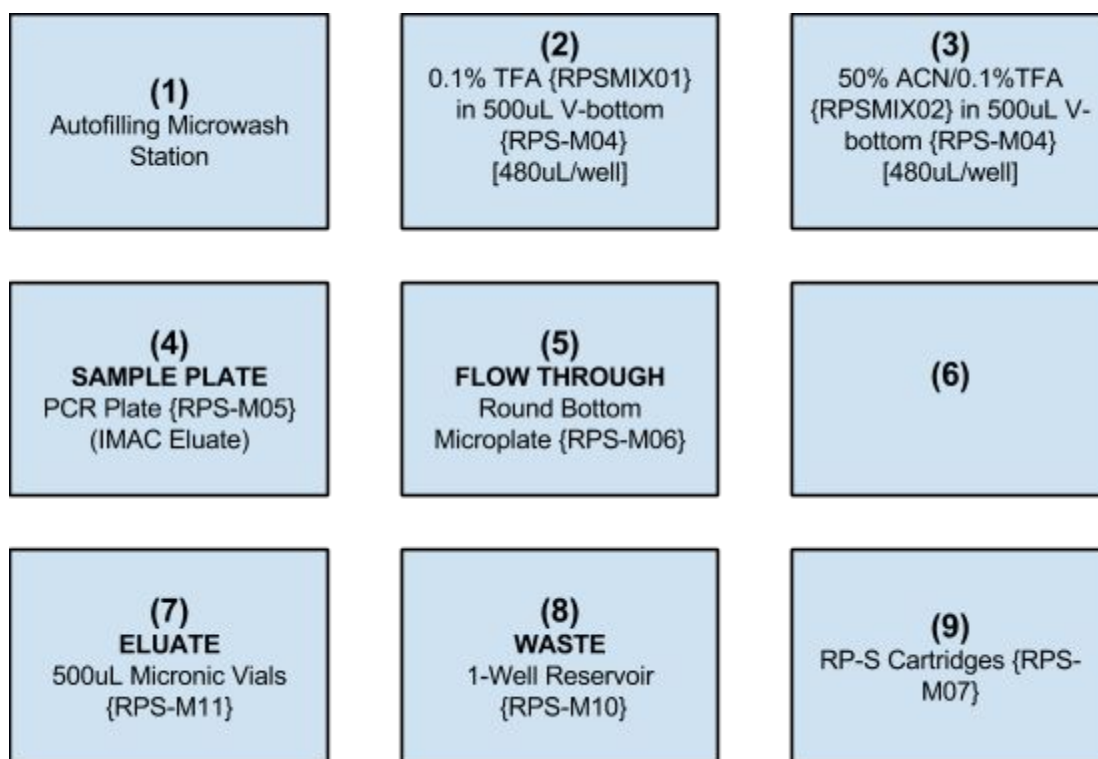
RPSMIX02 – 50% ACN/0.1% TFA
1. Measure 500mL of HPLC-grade water {RPS-M01} in a graduated cylinder and add to a 1L bottle. ❑
2. Measure 500mL of acetonitrile {RPS-M02} in a graduated cylinder and add to the bottle. ❑
3. Pipette 1mL Trifluoroacetic acid {RPS-M03} into the bottle. ❑

Procedure

1. Fill a 500uL V-bottom plate {RPS-M04} with 480uL of 0.1%TFA {RPSMIX01} [355uL required].
2. Fill a 500uL V-bottom plate { RPS-M04} with 480uL of 50% ACN/0.1% TFA {RPSMIX02} [350uL required].

3. Fill the Wash Station reservoir with 0.1% TFA {RPSMIX01} and ensure that the tubing is fully submerged. Check the waste container to see if the tubing leading into it is still above liquid level. If the container is full, dispose of the waste in the correct satellite waste container
4. Prepare AM-BRAVO for operation:
    4.1. On the AM-BRAVO Eva {IMAC-A01} load the device file "AssayMap Bravo_CF.dev". This file is located at C:\\VWorks Workspace\Device Files\.
    4.2. In the "Devices" page, click on "Agilent Bravo" and then "AM-EVA". Select "Initialize all devices".
    4.3. Open the protocol file "3.0_RP-S_Bind Wash Elute_CF_V4.pro". This file is located at C:\\VWorks Workspace\Protocol Files\AM-Bravo\IMAC\.
5. Assemble the deck of the LT-BRAVO according to the following layout:

Note: The **SAMPLE PLATE** in position 4 is the **IMAC ELUATE** plate from the previous protocol, **Automated IMAC Enrichment (IMAC).**

(1) Autofilling Microwash Station	**(2)** 0.1% TFA {RPSMIX01} in 500uL V-bottom {RPS-M04} [480uL/well]	**(3)** 50% ACN/0.1%TFA {RPSMIX02} in 500uL V-bottom {RPS-M04} [480uL/well]
(4) **SAMPLE PLATE** PCR Plate {RPS-M05} (IMAC Eluate)	**(5)** **FLOW THROUGH** Round Bottom Microplate {RPS-M06}	**(6)**
(7) **ELUATE** 500uL Micronic Vials {RPS-M11}	**(8)** **WASTE** 1-Well Reservoir {RPS-M10}	**(9)** RP-S Cartridges {RPS-M07}

6. On the AM-BRAVO, toggle to "Simulation is on" at the top of the screen from "Simulation is off".
    6.1. Press Start and the Run Configuration Wizard will pop up. Press Finish.
    6.2. A pop up entitled "Set Initial Values for Variables" will appear. Set the number of "CartridgeColumns" to the appropriate amount of sample columns. (RPS Automation Protocol Step 1).
    6.3. Change the values for the other parameters listed if necessary and press ok.
    6.4. The simulation will run and provide feedback on any warnings or errors that the protocol may encounter. If there are any **unknown** errors that come up, notify the key AM-BRAVO user and obtain help.
7. On the AM-BRAVO, toggle back to "Simulation is off". Follow steps 6.1 to 6.3 in order to run the protocol. (RPS Automation Protocol Steps 2-8).

8. When the protocol is finished, clear the deck.
    8.1. Dispose of waste in the "Waste" reservoir at the appropriate satellite accumulation station.
    8.2. Retain any labware that can be reused. Empty appropriately, rinse with water, and leave to dry.
9. Upon completion of the protocol, note the condition of samples and seal plates to be saved.
    9.1. No volume should remain in the **SAMPLE PLATE** in position 4. Seal this plate with foil {RPS-M09} and store at -80C.
    9.2. Place foil {RPS-M09} on the **FLOW THROUGH** plate and transfer to -80C.
    9.3. Create a **Balance** plate by pipetting 50uL of 50% ACN/0.1% TFA {RPSMIX02} into each vial of a Micronic Vials Loborack {RPS-M11}.
    9.4. Cover the RP-S **ELUATE** plate in position 7 with foil {RPS-M09}, vortex, spin down, and remove the seal.
    9.5. Cover both RP-S **ELUATE** and **Balance** plates with a breathable seal {RPS-M08}, then foil {RPS-M09}, and freeze at -80°C.
    9.6. Remove the foil seals from the RP-S **ELUATE** and **Balance** plate and speedvac to dryness.
        9.6.1. Once dry, keep peptides at 4°C if analyzing immediately or freeze at -80°C.

E. Appendix:

1. TIP Automation Steps (AssayMap-BRAVO)

1. Define Variables
    1.1. CartridgeColumns = 3
    1.2. PrepVolume = 25
    1.3. SampleRate = 0.033
    1.4. WWV = 25
    1.5. WashRate = 0.417
2. Water Wash 1
    2.1. Set head mode to all barrels
        2.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
        2.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
    2.2. Wash Tips with 240uL at Position 3 (Wash Station).
        2.2.1. Liquid class = AM_50uLperSec
        2.2.2. Mix cycles = 1
    2.3. Loop 3 times changing tips every 1 time.
    2.4. AM Aspirate 25uL from Position 3. (Water)
        2.4.1. Volume = WWV
        2.4.2. Distance from well bottom = 2
    2.5. AM Cartridges on from Position 9.
    2.6. Dispense to waste contents of tips to Position 1. (Wash Station)
        2.6.1. Liquid class = !AM_25uLperMin_0.42uLperSec
        2.6.2. Dispense flow rate =WashRate
        2.6.3. Distance from well bottom = 22

- 2.7. AM Cartridges off at Position 9.
- 2.8. Loop End.
3. Strip
    - 3.1. Set head mode to all barrels
        - 3.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
        - 3.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
    - 3.2. Loop 2 times changing tips every 1 time.
    - 3.3. AM Aspirate 25uL from Postion 7. (EDTA)
        - 3.3.1. Volume = PrepVolume
        - 3.3.2. Distance from well bottom = 0.5
    - 3.4. AM Cartridges on from Position 9.
    - 3.5. AM Dispense contents of tips to Position 4.
        - 3.5.1. Liquid class = AM_10uLperSec
        - 3.5.2. Dispense flow rate = SampleRate
        - 3.5.3. Distance from well bottom = 2
    - 3.6. AM Cartridges off at Position 9.
    - 3.7. Loop End.
    - 3.8. Wash Tips with 240uL at Position 3. (Wash Station)
        - 3.8.1. Liquid class = AM_50uLperSec
        - 3.8.2. Mix cycles = 3
    - 3.9. AM Dispense contents of tips to Position 1. (Wash Station)
4. Water Wash 2
    - 4.1. Set head mode to all barrels
        - 4.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
        - 4.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
    - 4.2. Wash Tips with 240uL at Position 1 (Wash Station).
        - 4.2.1. Liquid class = AM_50uLperSec
        - 4.2.2. Mix cycles = 1
    - 4.3. Loop 3 times changing tips every 1 time.
    - 4.4. AM Aspirate 25uL from Position 3. (Water)
        - 4.4.1. Volume = WWV
        - 4.4.2. Distance from well bottom = 2
    - 4.5. AM Cartridges on from Position 9.
    - 4.6. Dispense to waste contents of tips to Position 1. (Wash Station)
        - 4.6.1. Liquid class = !AM_25uLperMin_0.42uLperSec
        - 4.6.2. Dispense flow rate =WashRate
        - 4.6.3. Distance from well bottom = 22
    - 4.7. AM Cartridges off at Position 9.
    - 4.8. Loop End.
5. Reload
    - 5.1. Set head mode to all barrels
        - 5.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
        - 5.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
    - 5.2. Loop 2 times changing tips every 1 times
    - 5.3. AM Aspirate 25uL from Positon 8. (FeCl3)

    5.3.1. Volume = PrepVolume

    5.3.2. Distance from well bottom =0.5

  5.4. AM Cartridges on at Position 9.

  5.5. AM Dispense contents of tips to Position 5.

    5.5.1. Liquid class = AM_10uLperSec

    5.5.2. Dispense flow rate = SampleRate

    5.5.3. Distance from well bottom = 2

  5.6. AM Cartridges off at Position 9.

  5.7. Loop End.

  5.8. Wash Tips with 240uL at Position 1. (Wash Station)

    5.8.1. Liquid class = AM_50uLperSec

    5.8.2. Mix cycles = 3

  5.9. AM Dispense contents of tips to Position 1.

6. Water Wash 3

  6.1. Set head mode to all barrels

    6.1.1. task.Headmode="1,2,8,"+CartridgeColumns;

    6.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

  6.2. Wash Tips with 240uL at Position 3 (Wash Station).

    6.2.1. Liquid class = AM_50uLperSec

    6.2.2. Mix cycles = 1

  6.3. Loop 3 times changing tips every 1 time.

  6.4. AM Aspirate 25uL from Position 3. (Water)

    6.4.1. Volume = WWV

    6.4.2. Distance from well bottom = 2

  6.5. AM Cartridges on from Position 9.

  6.6. Dispense to waste contents of tips to Position 1. (Wash Station)

    6.6.1. Liquid class = !AM_25uLperMin_0.42uLperSec

    6.6.2. Dispense flow rate =WashRate

    6.6.3. Distance from well bottom = 22

  6.7. AM Cartridges off at Position 9.

  6.8. Loop End.

2. IMAC Automation Steps (AssayMap-BRAVO)

1. Define Variables

  1.1. CartridgeColumns = 12

  1.2. ElutionRate = 0.083

  1.3. ElutionVolume = 50

  1.4. EquilibrationRate = 0.417

  1.5. EquilibrationVolume = 100

  1.6. LoadLoops = 4

  1.7. NumberOfCupWashes = 2

  1.8. PrimingRate = 1.67

  1.9. SampleRate = 0.333

  1.10. SampleVolume = 175

1.11.   WashRate = 0.417

2.   Priming
 2.1.   Set head mode to all barrels
  2.1.1.   task.Headmode="1,2,8,"+CartridgeColumns;
  2.1.2.   This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
 2.2.   Wash tips with 240uL at Postion 1. (Wash Station)
  2.2.1.   Liquid class = AM_50uLperSec
  2.2.2.   Mix cycles = 1
 2.3.   AM Aspirate 100uL from Position 7. (Priming)
  2.3.1.   Volume = EquilibrationVolume
  2.3.2.   Liquid class = AM_100uLperSec
  2.3.3.   Distance from well bottom = 0.5
 2.4.   AM Cartridges on at Position 9.
 2.5.   AM Dispense contents of tips to Position 8. (Waste)
  2.5.1.   Liquid class = AM_25uLperSec
  2.5.2.   Dispense flow rate = PrimingRate
  2.5.3.   Distance from well bottom = 2
 2.6.   AM Cartridges off at Position 9.

3.   Sample Loading
 3.1.   Set head mode to all barrels
  3.1.1.   task.Headmode="1,2,8,"+CartridgeColumns;
  3.1.2.   This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
 3.2.   AM Aspirate 175uL from Position 4. (Sample Plate)
  3.2.1.   Volume = SampleVolume
  3.2.2.   Liquid class = CF_200uLperMin_10secDelay
  3.2.3.   Distance from well bottom = -3
 3.3.   AM Cartridges on from Position 9.
 3.4.   Loop 4 times changing tips every 1 time.
 3.5.   AM Dispense contents of tips to Position 4. (Sample Plate)
  3.5.1.   Liquid class = CF_20uLperMin_0.333uLperSec_1minDelay
  3.5.2.   Dispense flow rate = SampleRate
  3.5.3.   Distance from well bottom = -1
 3.6.   AM Aspirate 150uL from Position 4. (Sample Plate)
  3.6.1.   Liquid class = CF_20uLperMin_0.333uLperSec_1minDelay
  3.6.2.   Aspirate flow rate = SampleRate
  3.6.3.   Distance from well bottom = -2
 3.7.   Loop End.
 3.8.   AM Dispense contents of tips to Position 6. (Flow Through)
  3.8.1.   Liquid class = CF_20uLperMin_0.333uLperSec_1minDelay
  3.8.2.   Dispense flow rate = SampleRate
  3.8.3.   Distance from well bottom = 3
 3.9.   AM Cartridges off at Position 9.
 3.10.   Wash tips with 240uL at Position 1. (Wash Station)
  3.10.1.   Liquid class = AM_50uLperSec

3.10.2.   Mix cycles = 3

3.10.3.   Distance from well bottom = 20

3.11.   AM Dispense contents of tips to Position 1. (Wash Station)

      3.11.1.1.   Liquid class = CF_200uLperMin_10secDelay

      3.11.1.2.   Dispense flow rate = EquilibrationRate

      3.11.1.3.   Distance from well bottom = 20

3.12.   Wait for the user to press GO.

    3.12.1.   Text: Put 80%ACN/0.1%TFA in Pos 3 and 500mM K2HPO4 at Pos 2, then press GO.

4.   Cup Washing

   4.1.   Set head mode to all barrels

     4.1.1.   task.Headmode="1,2,8,"+CartridgeColumns;

     4.1.2.   This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

   4.2.   Loop 2 times changing tips every 1 time.

     4.2.1.   Number of times to loop =NumberOfCupWashes

   4.3.   AM Aspirate 50uL from Position 3. (Binding Buffer)

     4.3.1.   Volume = ElutionVolume

     4.3.2.   Liquid class = CF_200uLperMin_10secDelay

     4.3.3.   Distance from well bottom = 2

   4.4.   AM Dispense contents of tips to Position 9. (IMAC Cartridges)

     4.4.1.   Liquid class = CF_200uLperMin_10secDelay

     4.4.2.   DIstance from well bottom = -13

   4.5.   AM Aspirate 75uL from Position 9. (IMAC Cartridges)

     4.5.1.   Liquid class = CF_200uLperMin_10secDelay

     4.5.2.   Distance from well bottom = -17

   4.6.   AM Cartridges off at Position 9.

   4.7.   AM Dispense contents of tips to Position 6. (Flow Through)

     4.7.1.   Liquid class = CF_200uLperMin_10secDelay

     4.7.2.   Distance from well bottom = 3

   4.8.   Wash tips with 240uL at Position 1. (Wash Station)

     4.8.1.   Liquid class = AM_50uLperSec

     4.8.2.   Mix cycles = 1

     4.8.3.   Distance from well bottom = 20

   4.9.   Loop End.

5.   Internal Cartridge Washing

   5.1.   Set head mode to all barrels

     5.1.1.   task.Headmode="1,2,8,"+CartridgeColumns;

     5.1.2.   This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

   5.2.   AM Aspirate 50uL from Position 1. (Binding Buffer)

     5.2.1.   Liquid class = CF_200uLperMin_10secDelay

     5.2.2.   Aspirate flow rate =ElutionRate

     5.2.3.   Distance from well bottom = 2

   5.3.   AM Cartridges on from Position 9.

   5.4.   AM Dispense contents of tips to Position 4. (Flow Through)

     5.4.1.   Liquid class = CF_20uLperMin_0.333uLperSec_1MinDelay

5.4.2. Distance from well bottom = 3
5.5. AM Cartridges off at Position 9.
5.6. Wash tips with 240uL at Position 1. (Wash Station)
5.6.1. Liquid class = AM_50uLperSec
5.6.2. Mix cycles = 2
5.6.3. Distance from well bottom = 20

6. Stringent Syringe Washing
6.1. Set head mode to all barrels
6.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
6.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
6.2. Loop 2 times changing tips every 1 time.
6.2.1. Number of times to loop =NumberOfCupWashes
6.3. AM Aspirate 50uL from Position 2. (Elution Buffer)
6.3.1. Liquid class = CF_200uLperMin_10secDelay
6.3.2. Distance from well bottom = 2
6.4. AM Dispense contents of tips to Position 8. (Waste)
6.4.1. Liquid class = CF_200uLperMin_10secDelay
6.4.2. Dispense flow rate = PrimingRate
6.4.3. Distance from well bottom = 7
6.5. Wash Tips with 0uL at Position 3. (Wash Station)
6.5.1. Liquid class = AM_10uLperSec
6.5.2. Mix cycles = 0
6.5.3. Distance from well bottom = 20
6.6. Loop End.

7. Elution
7.1. Set head mode to all barrels
7.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
7.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
7.2. AM Aspirate 50uL from Position 2. (Elution Buffer)
7.2.1. Volume =ElutionVolume
7.2.2. Liquid class = CF_200uLperMin_10SecDelay
7.2.3. Distance from well bottom = 1
7.3. Wash tips with 0uL at Position 1. (Wash Station)
7.3.1. Liquid class = AM_10uLperSec
7.3.2. Distance from well bottom = 20
7.4. AM Cartridges on at Position 9.
7.5. AM Dispense contents of tips to Position 5. (Eluate)
7.5.1. Liquid class = CF_20uLperMin_0.333uLperSec_1MinDelay
7.5.2. Dispense flow rate =ElutionRate
7.5.3. DIstance from well bottom = 2
7.6. Wash tips with 0uL at Position 1. (Wash Station)
7.6.1. Liquid class = AM_2_5sec Delay
7.6.2. Mix cycles = 0
7.6.3. Distance from well bottom = 22

7.7.   AM Cartridges off at Position 9.
7.8.   Wash tips with 240uL at Position 3. (Wash Station)
    7.8.1.   Liquid class = AM_50uLperSec
    7.8.2.   Mix cycles = 1
    7.8.3.   Distance from well bottom = 20
7.9.   Move above location 1.
7.10.   Dispense to waste contents of tips at Position 1. (Wash Station)
    7.10.1.   Distance from well bottom = 25

3.   RPS Automation Steps (AssayMap-BRAVO)

1.   Define Variables
    1.1.   CartridgeColumns = 12
    1.2.   ElutionRate = 0.083
    1.3.   ElutionVolume = 50
    1.4.   EquilibrationRate = 0.417
    1.5.   EquilibrationVolume = 50
    1.6.   NumberOfCupWashes = 3
    1.7.   PrimingRate = 5
    1.8.   SampleRate = 0.033
    1.9.   SampleVolume = 50
    1.10.   WashLoops = 3
    1.11.   WashRate = 0.417
    1.12.   WashVolume = 50

2.   Priming
    2.1.   Set head mode to all barrels
        2.1.1.   task.Headmode="1,2,8,"+CartridgeColumns;
        2.1.2.   This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
    2.2.   Wash tips with 240uL at Position 1. (Wash Station)
        2.2.1.   Liquid class = AM_50uLperSec
        2.2.2.   Mix cycles = 1
    2.3.   AM Aspirate 150uL from Position 3. (Elution Buffer)
        2.3.1.   Liquid class = AM_100uLperSec
        2.3.2.   Distance from well bottom = 3
    2.4.   AM Cartridges on at Position 9.
    2.5.   AM Dispense contents of tips to Position 8. (Organic Waste)
        2.5.1.   Liquid class = AM_25uLperSec
        2.5.2.   Dispense flow rate = PrimingRate
        2.5.3.   Distance from well bottom = 5
    2.6.   AM Cartridges off at Position 9.

3.   Equilibration
    3.1.   Set head mode to all barrels
        3.1.1.   task.Headmode="1,2,8,"+CartridgeColumns;

3.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

3.2. Wash tips by emptying contents of tips to Position 1. (Wash Station)

3.2.1. Liquid class = 2_5sec Delay

3.2.2. Distance from well bottom = 20

3.3. Move above location 1.

3.4. Dispense to waste contents of tips at Position 1. (Wash Station)

3.4.1. Distance from well bottom = 25

3.5. AM Aspirate 50uL from Position 2. (Utility Buffer)

3.5.1. Volume = EquilibrationVolume

3.5.2. Liquid class = CF_200uLperMin_10secDelay

3.5.3. Distance from well bottom = 2

3.6. AM Cartridges on from Position 9.

3.7. Dispense to waste contents of tips at Position 1. (Wash Station)

3.7.1. Liquid class = !AM_10uLperMin_0.167uLperSec

3.7.2. Dispense flow rate =EquilibrationRate

3.7.3. Distance from well bottom = 22

3.8. Wash tips with 0uL at Position 1. (Wash Station)

3.8.1. Liquid class = AM_2_5sec Delay

3.8.2. Mix cycles = 0

3.8.3. Distance from well bottom = 22

3.9. AM Cartridges off at Position 9.

3.10. Wash tips with 240uL at Position 1. (Wash Station)

3.10.1. Liquid class = AM_50uLperSec

3.10.2. Mix cycles = 1

3.10.3. Distance from well bottom = 20

3.11. Move above location 1.

3.12. Dispense to waste contents of tips at Position 1. (Wash Station)

3.12.1. Distance from well bottom = 25

4. Sample Loading

4.1. Set head mode to all barrels

4.1.1. task.Headmode="1,2,8,"+CartridgeColumns;

4.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

4.2. AM Aspirate 50uL from Position 4. (Sample Plate)

4.2.1. Volume =SampleVolume

4.2.2. Liquid class = CF_200uLperMin_10SecDelay

4.2.3. Distance from well bottom = 0

4.3. AM Cartridges on from Position 9.

4.4. AM Dispense contents of tips to Position 5. (Flow Through)

4.4.1. Liquid class = AM_10uLperSec

4.4.2. Dispense flow rate = SampleRate

4.4.3. Distance from well bottom = 2

4.5. Wash tips with 0uL at Position 3. (Wash Station)

4.5.1. Liquid class = AM_2_5sec Delay

4.5.2. Mix cycles = 0

4.5.3. Distance from well bottom = 22

4.6. AM Aspirate 5uL from Position 2. (Utility Buffer)
    4.6.1. Liquid class = AM_10uLperSec
    4.6.2. Aspirate flow rate =SampleRate
    4.6.3. Distance from well bottom = 2
4.7. AM Cartridges off at Position 9.
4.8. AM Dispense contents of tips to Position 5. (Flow Through)
    4.8.1. Distance from well bottom = 2
4.9. Wash tips with 240uL at Position 3. (Wash Station)
    4.9.1. Liquid class = AM_50uLperSec
    4.9.2. Mix cycles = 1
    4.9.3. Distance from well bottom = 20
4.10. Move above location 1.
4.11. Dispense to waste contents of tips at Position 1. (Wash Station)
    4.11.1. Distance from well bottom = 25

5. Cup Washing
    5.1. Set head mode to all barrels
        5.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
        5.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
    5.2. Loop 3 times changing tips every 1 time.
        5.2.1. Number of times to loop =NumberOfCupWashes
    5.3. AM Aspirate 50uL from Position 2. (Utility Buffer)
        5.3.1. Liquid class = CF_200uLperMin_10SecDelay
        5.3.2. Distance from well bottom = 2
    5.4. AM Dispense contents of tips to Position 9. (RP-S Cartridges)
        5.4.1. Liquid class = CF_200uLperMin_10SecDelay
        5.4.2. DIstance from well bottom = -13
    5.5. AM Aspirate 75uL from Position 9. (RP-S Cartridges)
        5.5.1. Liquid class = CF_200uLperMin_10SecDelay
        5.5.2. Distance from well bottom = -17
    5.6. AM Cartridges off at Position 9.
    5.7. Dispense to waste contents of tips to Position 1. (Wash Station)
        5.7.1. Distance from well bottom = 25
    5.8. Wash tips with 240uL at Position 1. (Wash Station)
        5.8.1. Liquid class = AM_50uLperSec
        5.8.2. Mix cycles = 1
        5.8.3. Distance from well bottom = 20
    5.9. Move above location 1.
    5.10. Dispense to waste contents of tips to Position 1. (Wash Station)
        5.10.1. Distance from well bottom = 25
    5.11. Loop End.

6. Internal Cartridge Washing
    6.1. Set head mode to all barrels
        6.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
        6.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

6.2. Loop 3 times changing tips every 1 time.
    6.2.1. Number of times to loop = WashLoops
6.3. AM Aspirate 50uL from Position 2. (Utility Buffer)
    6.3.1. Volume =WashVolume
    6.3.2. Liquid class = CF_200uLperMin_10SecDelay
    6.3.3. Aspirate flow rate =ElutionRate
    6.3.4. Distance from well bottom = 2
6.4. AM Cartridges on from Position 9.
6.5. AM Dispense contents of tips to Position 4. (Flow Through)
    6.5.1. Liquid class = AM_10uLperSec
    6.5.2. Dispense flow rate = ElutionRate
    6.5.3. Distance from well bottom = 2
6.6. AM Cartridges off at Position 9.
6.7. Loop End.
6.8. Wash tips with 240uL at Position 1. (Wash Station)
    6.8.1. Liquid class = AM_50uLperSec
    6.8.2. Mix cycles = 1
    6.8.3. Distance from well bottom = 20
6.9. Move above location 1.
6.10. Dispense to waste contents of tips to Position 1. (Wash Station)
    6.10.1. Distance from well bottom = 25

7. Stringent Syringe Washing
    7.1. Set head mode to all barrels
        7.1.1. task.Headmode="1,2,8,"+CartridgeColumns;
        7.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.
    7.2. Loop 3 times changing tips every 1 time.
        7.2.1. Number of times to loop =NumberOfCupWashes
    7.3. AM Aspirate 50uL from Position 3. (Elution Buffer)
        7.3.1. Liquid class = CF_200uLperMin_10SecDelay
        7.3.2. Distance from well bottom = 2
    7.4. AM Dispense contents of tips to Position 8. (Organic Waste)
        7.4.1. Liquid class = CF_200uLperMin_10SecDelay
        7.4.2. Distance from well bottom = 7
    7.5. Wash Tips with 0uL at Position 1. (Wash Station)
        7.5.1. Liquid class = AM_10uLperSec
        7.5.2. Mix cycles = 0
        7.5.3. Distance from well bottom = 20
    7.6. Move above location 1.
    7.7. Dispense to waste contents of tips to Position 1. (Wash Station)
        7.7.1. Distance from well bottom = 25
    7.8. Loop End.

8. Elution
    8.1. Set head mode to all barrels
        8.1.1. task.Headmode="1,2,8,"+CartridgeColumns;

8.1.2. This script can be used in "Advanced Settings" in conjunction with "Define Variables" to set the number of "Cartridge Columns" to the appropriate number.

8.2. AM Aspirate 50uL from Position 3. (Elution Buffer)

8.2.1. Volume =ElutionVolume

8.2.2. Liquid class = CF_200uLperMin_10SecDelay

8.2.3. Distance from well bottom = 1

8.3. Wash tips with 0uL at Position 1. (Wash Station)

8.3.1. Liquid class = AM_10uLperSec

8.3.2. Distance from well bottom = 20

8.4. AM Cartridges on at Position 9.

8.5. AM Dispense contents of tips to Position 7. (Eluate)

8.5.1. Liquid class = AM_10uLperSec

8.5.2. Dispense flow rate =ElutionRate

8.5.3. DIstance from well bottom = 2

8.6. Wash tips with 0uL at Position 1. (Wash Station)

8.6.1. Liquid class = AM_2_5sec Delay

8.6.2. Mix cycles = 0

8.6.3. Distance from well bottom = 22

8.7. AM Cartridges off at Position 9.

8.8. Wash tips with 240uL at Position 1. (Wash Station)

8.8.1. Liquid class = AM_50uLperSec

8.8.2. Mix cycles = 1

8.8.3. Distance from well bottom = 20

8.9. Move above location 1.

8.10. Dispense to waste contents of tips at Position 1. (Wash Station)

8.10.1. Distance from well bottom = 25